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<b>(21) International Application Number:</b> PCT/EP97/05578 <b>(22) International Filing Date:</b> 30 September 1997 (30.09.97)  <b>(30) Priority Data:</b> 9620795.6 5 October 1996 (05.10.96) GB  <b>(71) Applicant (for all designated States except US):</b> SMITHK-LINE BEECHAM BIOLOGICALS S.A. [BE/BE]; Rue de l'Institut 89, B-1330 Rixensart (BE).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> GARCON, Nathalie [FR/BE]; SmithKline Beecham Biologicals S.A., Rue de l'Institut 89, B-1330 Rixensart (BE). FRIEDE, Martin [GB/BE]; SmithKline Beecham Biologicals S.A., Rue de l'Institut 89, B-1330 Rixensart (BE).  <b>(74) Agent:</b> DALTON, Marcus, Jonathan, William; SmithKline Beecham plc, Corporate Intellectual Property, Two New Horizons Court, Brentford, Middlesex TW8 9EP (GB).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> VACCINES  <b>(57) Abstract</b>  The invention relates to a vaccine composition comprising alum, an antigen, an immunologically active saponin fraction and a sterol.		

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### Vaccines

The present invention relates to novel vaccine formulations, to methods of their production and to their use in medicine. In particular, the present invention  
5 relates to vaccines containing alum, an antigen, an immunologically active fraction derived from the bark of Quillaja Saponaria Molina such as QS21, and a sterol.

Immunologically active saponin fractions having adjuvant activity derived from the bark of the South American tree Quillaja Saponaria Molina are known in the art. For example QS21, also known as QA21, an Hplc purified fraction from the  
10 Quillaja Saponaria Molina tree and it's method of its production is disclosed (as QA21) in US patent No. 5,057,540. Quillaja saponin has also been disclosed as an adjuvant by Scott et al, Int. Archs. Allergy Appl. Immun., 1985, 77, 409. However, the use of QS21 as an adjuvant is associated with certain disadvantages. For example when QS21 is injected into a mammal as a free molecule it has been observed that  
15 necrosis, that is to say, localised tissue death, occurs at the injection site.

WO 96/33739 discloses vaccine formulations comprising an antigen, an immunologically active fraction derived from the bark of Quillaja Saponaria Molina such as QS21, and a sterol.

It has now surprisingly been found that incorporation of alum in vaccine  
20 formulations containing MPL, QS21 and SUV enhances both humoral and cellular responses and that vaccine formulations containing MPL, QS21, SUV and alum are non-toxic with a good reactogenicity profile and have enhanced adjuvant activity. In addition, the combined adjuvant appears to favour TH1 responses.

In a first aspect the invention provides a vaccine adjuvant comprising alum, an  
25 immunologically active saponin fraction, and a sterol. By the term 'alum' is meant aluminium hydroxide or aluminium phosphate.

Preferably the adjuvant compositions of the invention contain the immunologically active saponin fraction in substantially pure form. Preferably the compositions of the invention contain QS21 in substantially pure form, that is to say,  
30 the QS21 is at least 90% pure, preferably at least 95% pure and most preferably at least 98% pure.

Other immunologically active saponin fractions useful in compositions of the invention include QA17/QS17. Compositions of the invention comprising QS21 and cholesterol show decreased reactogenicity when compared to compositions in which the cholesterol is absent, while the adjuvant effect is maintained. In addition it is  
5 known that QS21 degrades under basic conditions where the pH is about 7 or greater. A further advantage of the present compositions is that the stability of QS21 to base-mediated hydrolysis is enhanced in formulations containing cholesterol.

Preferred sterols include  $\beta$ -sitosterol, stigmasterol, ergosterol, ergocalciferol and cholesterol. These sterols are well known in the art, for example cholesterol is  
10 disclosed in the Merck Index, 11th Edn., page 341, as a naturally occurring sterol found in animal fat. Most preferably the sterol is cholesterol.

Preferred compositions of the invention are those forming a liposome structure, that is to say small unilammellar vesicles (SUV). Compositions where the sterol/immunologically active saponin fraction forms an ISCOM structure also form  
15 an aspect of the invention.

The ratio of QS21 : sterol will typically be in the order of 1 : 100 to 1 : 1 weight to weight. Preferably excess sterol is present, the ratio of QS21 : sterol being at least 1 : 1 w/w. Typically for human administration QS21 and sterol will be present in a vaccine in the range of about 1  $\mu$ g to about 100  $\mu$ g, preferably about 10  $\mu$ g to  
20 about 50  $\mu$ g per dose of QS21.

The liposomes preferably contain a neutral lipid, for example phosphatidylcholine, which is preferably non-crystalline at room temperature, for example egg yolk phosphatidylcholine, dioleoyl phosphatidylcholine or dilauryl phosphatidylcholine. The liposomes may also contain a charged lipid which increases  
25 the stability of the liposome-QS21 structure for liposomes composed of saturated lipids. In these cases the amount of charged lipid is preferably 1-20% w/w, most preferably 5-10%. The ratio of sterol to phospholipid is 1-50% (mol/mol), most preferably 20-25%.

Particularly preferred and advantageous compositions of the invention contain  
30 MPL (3-deacylated mono-phosphoryl lipid A, also known as 3D-MPL). 3D-MPL is known from GB 2 220 211 (Ribi) as a mixture of 3 types of De-O-acylated

monophosphoryl lipid A with 4, 5 or 6 acylated chains and is manufactured by Ribi Immunochem, Montana. A preferred form is disclosed in International Patent Application 94/21292.

Suitable compositions of the invention are those wherein liposomes are initially prepared without MPL, and MPL is then added, preferably as 100 nm particles. The MPL is therefore not contained within the vesicle membrane (known as MPL out). Compositions where the MPL is contained within the vesicle membrane (known as MPL in) also form an aspect of the invention. The antigen can be contained within the vesicle membrane or contained outside the vesicle membrane. Preferably soluble antigens are outside and hydrophobic or lipidated antigens are either contained within or outside the membrane.

In a preferred aspect of the invention, liposomes/SUV are first added to the QS21 and then mixed with alum which results in a significant proportion of the QS21 binding to the alum (via interaction through the liposomes). Such a formulation, when injected, is expected to result in a slower release of QS21 to the body, due to a depot effect of the alum, than if the QS21 was free or in un-fixed liposomes. The formulation containing MPL, QS21, SUV and alum are particularly advantageous as they are non-toxic and highly immunogenic.

Preferably the vaccine formulations will contain an antigen or antigenic composition capable of eliciting an immune response against a human or animal pathogen. In a first aspect the present invention therefore provides a vaccine composition comprising alum, an antigen, an immunologically active saponin fraction and a sterol.

Antigen or antigenic compositions known in the art can be used in the compositions of the invention, including polysaccharide antigens, antigen or antigenic compositions derived from HIV-1, (such as gp120 or gp160), any of Feline Immunodeficiency virus, human or animal herpes viruses, such as gD or derivatives thereof or Immediate Early protein such as ICP27 from HSV1 or HSV2, cytomegalovirus (especially human) (such as gB or derivatives thereof), Varicella Zoster Virus (such as gpI, II or III), or from a hepatitis virus such as hepatitis B virus for example Hepatitis B Surface antigen or a derivative thereof, hepatitis A virus,

hepatitis C virus and hepatitis E virus, or from other viral pathogens, such as Respiratory Syncytial virus (for example HSRV F and G proteins or immunogenic fragments thereof disclosed in US Patent 5,149,650 or chimeric polypeptides containing immunogenic fragments from HSRV proteins F and G, eg FG glycoprotein  
5 disclosed in US Patent 5,194,595), antigens derived from meningitis strains such as meningitis A, B and C, Streptococcus Pneumonia, human papilloma virus, Influenza virus, Haemophilus Influenza B (Hib), Epstein Barr Virus (EBV), or derived from bacterial pathogens such as Salmonella, Neisseria, Borrelia (for example OspA or OspB or derivatives thereof), or Chlamydia, or Bordetella for example P.69, PT and  
10 FHA, or derived from parasites such as plasmodium or toxoplasma.

HSV Glycoprotein D (gD) or derivatives thereof is a preferred vaccine antigen. It is located on the viral membrane, and is also found in the cytoplasm of infected cells (Eisenberg R.J. et al; J of Virol 1980 35 428-435). It comprises 393 amino acids including a signal peptide and has a molecular weight of approximately  
15 60 kD. Of all the HSV envelope glycoproteins this is probably the best characterised (Cohen et al J. Virology 60 157-166). In vivo it is known to play a central role in viral attachment to cell membranes. Moreover, glycoprotein D has been shown to be able to elicit neutralising antibodies in vivo and protect animals from lethal  
20 challenge. A truncated form of the gD molecule is devoid of the C terminal anchor region and can be produced in mammalian cells as a soluble protein which is exported into the cell culture supernatant. Such soluble forms of gD are preferred. The production of truncated forms of gD is described in EP 0 139 417. Preferably the gD is derived from HSV-2. An embodiment of the invention is a truncated HSV-2 glycoprotein D of 308 amino acids which comprises amino acids 1 through 306  
25 naturally occurring glycoprotein with the addition Asparagine and Glutamine at the C terminal end of the truncated protein devoid of its membrane anchor region. This form of the protein includes the signal peptide which is cleaved to allow for the mature soluble 283 amino acid protein to be secreted from a host cell.

In another aspect of the invention, Hepatitis B surface antigen is a preferred  
30 vaccine antigen.

As used herein the expression 'Hepatitis B surface antigen' or 'HBsAg' includes any HBsAg antigen or fragment thereof displaying the antigenicity of HBV surface antigen. It will be understood that in addition to the 226 amino acid sequence of the HBsAg antigen (see Tiollais et al, Nature, 317, 489 (1985) and references therein) HBsAg as herein described may, if desired, contain all or part of a pre-S sequence as described in the above references and in EP-A- 0 278 940. In particular the HBsAg may comprise a polypeptide comprising an amino acid sequence comprising residues 12-52 followed by residues 133-145 followed by residues 175-400 of the L-protein of HBsAg relative to the open reading frame on a Hepatitis B virus of ad serotype (this polypeptide is referred to as L\*; see EP 0 414 374). HBsAg within the scope of the invention may also include the pre-S1-preS2-S polypeptide described in EP 0 198 474 (Endotronics) or close analogues thereof such as those described in EP 0 304 578 (Mc Cormick and Jones). HBsAg as herein described can also refer to mutants, for example the 'escape mutant' described in WO 91/14703 or European Patent Application Number 0 511 855A1, especially HBsAg wherein the amino acid substitution at position 145 is to arginine from glycine.

Normally the HBsAg will be in particle form. The particles may comprise for example S protein alone or may be composite particles, for example (L\*,S) where L\* is as defined above and S denotes the S-protein of HBsAg. The said particle is advantageously in the form in which it is expressed in yeast.

The preparation of hepatitis B surface antigen S-protein is well documented. See for example, Harford et al (1983) in Develop. Biol. Standard 54, page 125, Gregg et al (1987) in Biotechnology, 5, page 479, EP 0 226 846, EP 0 299 108 and references therein.

In another embodiment, the vaccine antigen is an RSV antigen. In particular an F/G antigen. US patent 5194595 (Upjohn) describes chimeric glycoproteins containing immunogenic segments of the F and G glycoproteins of RSV and suggests that such proteins can be expressed from a variety of systems including bacterial, yeast, mammalian (eg CHO cells) and insect cells (using for example a baculovirus).

Wathen et al (J. Gen. Virol. 1989, 70, 2625-2635) describes a particular RSV FG chimeric glycoprotein expressed using a baculovirus vector consisting of amino acids 1-489 of the F protein linked to amino acids 97-279 of the G protein.

5 The formulations within the scope of the invention may also contain an anti-tumour antigen and be useful for immunotherapeutically treating cancers.

Vaccine preparation is generally described in New Trends and Developments in Vaccines, edited by Voller et al., University Park Press, Baltimore, Maryland, U.S.A. 1978. Encapsulation within liposomes is described, for example, by Fullerton, U.S. Patent 4,235,877. Conjugation of proteins to macromolecules is disclosed, for  
10 example, by Likhite, U.S. Patent 4,372,945 and by Armor et al., U.S. Patent 4,474,757.

The amount of protein in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccines. Such amount will vary depending upon which specific immunogen  
15 is employed and how it is presented. Generally, it is expected that each dose will comprise 1-1000 mcg of protein, preferably 2-100 mcg, most preferably 4-40 mcg. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of appropriate immune responses in subjects. Following an initial vaccination, subjects may receive one or several booster immunisation  
20 adequately spaced.

The formulations of the present invention may be used for both prophylactic and therapeutic purposes.

Accordingly in a further aspect, the invention therefore provides use of a vaccine composition of the invention for the treatment of human patients. The  
25 invention provides a method of treatment comprising administering an effective amount of a vaccine of the present invention to a patient. In particular, the invention provides a method of treating viral, bacterial, parasitic infections or cancer which comprises administering an effective amount of a vaccine of the present invention to a patient.

30 The following examples and data illustrates the invention.



**Example 1 Preparation of vaccine containing alum, SUV MPL and QS21****1.1 Method of preparation of SUV**

5 A mixture of lipid (such as phosphatidylcholine either from egg-yolk or synthetic) and cholesterol in organic solvent, is dried down under vacuum (or alternatively under a stream of inert gas). An aqueous solution (such as phosphate buffered saline) is then added, and the vessel agitated until all the lipid is in suspension. This suspension is then microfluidised until the liposome size is reduced  
10 to 100 nm, and then sterile filtered through a 0.2  $\mu$ m filter. Extrusion or sonication could replace this step. Typically the cholesterol: phosphatidylcholine ratio is 1:4 (w/w), and the aqueous solution is added to give a final cholesterol concentration of 5 to 50 mg/ml.

15 1.2 Antigen (1-500  $\mu$ g, preferably 10-100  $\mu$ g) is added to alum eg (aluminium hydroxide or aluminium phosphate) (100-500  $\mu$ g) in water. The volume of water is chosen so that the volume of the final formulation is 500  $\mu$ l. After incubating for 15-30 minutes, 50  $\mu$ g of MPL is added in the form of small-particle MPL (WO94/21292). The MPL is left to adsorb onto the alum for 15-30 minutes at room temperature. 10-  
20 times concentrated phosphate buffered saline (1.5 M sodium chloride, 0.5M sodium phosphate pH 7.5) is then added in such a volume so as to render the final formulation isotonic. This formulation is incubated at room temperature for 15-30 minutes.

QS21 (50  $\mu$ g) is then added to SUV (containing between 50 and 250  $\mu$ g cholesterol). This mixture is added to the above alum/antigen/MPL/buffer mixture. If  
25 required a bacteriostatic such as thiomersal is added (50  $\mu$ g).

**Example 2**

Table 1 shows the binding of QS21 to alum in the presence and absence of  
30 liposomes containing 25% (w/w) in dioleoyl phosphatidylcholine, and using a five-fold excess of cholesterol over QS21.

Formulation	SUV	$\mu$ g QS21 bound
500 $\mu$ g Alum+50 $\mu$ g QS21	0	< 10
500 $\mu$ g Alum+50 $\mu$ g QS21	250 $\mu$ g chol + 1 mg DOPC	> 40

In order to increase the binding of QS21 to alum, the quantity of liposomes can be decreased. This decreases the cholesterol:QS21 ratio, however it has been shown that the QS21 remains non-toxic for cholesterol:QS21 ratios of 1:1 and greater.

- 5 Table 2 shows that if the quantity of alum is decreased (from 500 µg to 100 µg) the quantity of QS21 that is bound decreases significantly, and the quantity of MPL that is bound also decreases. By adding less liposomes, yet maintaining a cholesterol:QS21 ratio of 1:1 or greater, increased quantities of QS21 and MPL can be bound to the alum.

10

Formulation	Chol/QS21	µg QS21 bound	µg MPL bound
500 µg alum + 50 µg QS21 + 50 µg MPL	5/1	42	>48
100 µg alum + 50 µg QS21 + 50 µg MPL	5/1	17	>40
100 µg alum + 50 µg QS21 + 50 µg MPL	2/1	30	>45
100 µg alum + 50 µg QS21 + 50 µg MPL	1/1	40	>45

### Example 3

- 15 The adjuvant effect of a combination of antigen (gD2t from Herpes Simplex Virus-2 - expressed in CHO cells and comprises 283 amino acid from the mature N-terminal of the mature glycoprotein) with MPL and QS21 in combination with liposomes was tested with and without alum. The formulations were tested in African Green Monkeys.

- 20 African Green Monkeys were immunised twice (0, 28 days) with 20 µg gD2t plus 50 µg MPL plus 50 µg QS21 with or without liposomes (250 µg cholesterol plus 1 mg DOPC) and with or without 500 µg alum. On day 42 the immune response was analysed.

The results are outlined below in figures 1 to 4.

- 25 The humoral response was measured as IgG against the gD protein. Figure 1 shows that the combination of MPL+QS21+SUV+alum induced higher titres than in

the absence of alum. Figure 2 shows that the formulation of the invention provided the superior antigen specific proliferation.

The data shows that incorporation of aluminium hydroxide in vaccine formulations containing MPL and QS21 and SUV enhances both the humoral and cellular responses. This is an unexpected finding since it is generally accepted that aluminium as an adjuvant tends to favour Th2 type responses, yet the results presented here demonstrate that the response contains a significant Th1 component which is not depressed by the addition of alum.

The formulation containing MPL and QS21 and SUV and alum is non-toxic and highly immunogenic.

#### **Example 4    Production of RSV FG CHO cell derived proteins**

The plasmid pEE14-FG contains a chimeric construct comprising of a fusion between amino acid sequences of F (1-525) and G (69-298) and was received from a collaboration with A. BOLLEN (ULB/CRI, Belgium). This FG fusion protein contains a total of 755 amino acids. It starts at the N-terminal signal sequence of F and lacks the C-terminal transmembrane domain (525-574) -anchor domain- of F glycoprotein. Then, followed the extracellular region of G glycoprotein, without the amino-terminal region that contains the Signal/Anchor domain of G, a typical class II glycoprotein.

The pEE14-FG expression plasmid was generated by the insertion of the FG coding sequence from pNIV2857 (A. Bollen, ULB/CRI, Belgium) as an Asp7181 (blunt) 5' - HindIII (blunt) 3' restriction fragment (2188 bp) into the SmaI site of pEE14 (Celltech). A Kozak sequence in lieu of the FG start ATG was generated into the pNIV 2857 construction as follows:

```

pEE14---ccc gtacc  ATG GAG -----x-----CAG TAG aagct ggg ---pEE14
          (SmaI)   Met1                Gln(298)Stop
                Asp718I(klenow)                HindIII(klenow)
30                5'F(1-525)-----x-----G(69-298)3'

```

The F sequence in pEE14-FG is from SS2 RSV strain, and was kindly made available by Dr. PRINGLE as a cDNA construct in a Vaccinia vector (Baybutt and Pringle, J.Gen.Virol., 1987, 2789-2796). The G sequence is from A2 RSV strain and was generated from a recombinant G Vaccinia virus obtained from Dr. G. WERTZ  
5 (Alabama, USA).

#### **CHO K1 transfection and stable FG protein expression.**

CHO K1 cells derived from MCB O24M (Celltech) were transfected with 20 ug of pEE14-FG plasmid DNA twice CsCl purified using the Ca-phosphate -glycerol  
10 transfection procedure. Cell clones were selected according to the procedure of the GS (glutamine synthetase ) expression system (Crocetti et al BioTechn., 1990, Vol8, 662) and amplified in the presence of 25 micro molar methionine sulfoximine (MSX) in G MEM medium containing no glutamine and supplemented with 10% dialyzed FBS (Foetal Bovine Serum). Following transfection, 39 MSX resistant clones were  
15 screened in 24-well plates and their supernatants were tested for secretion of the FG fusion protein. All transfectants proved to be positive for F antigen expression using a specific 'Sandwich' ELISA assay (i.e. rabbit polyclonal anti FG serum / Antigen / mAB19). Monoclonal antibody 19 recognises a conformational F1 - epitope and is neutralising.

20 The 3 best FG-producer clones (n° 7, 13 and 37) were single-cell subcloned in a limiting dilution assay using 0.07 cells per well in a 96-well plates. A total of 59 positive subclones were obtained and the 16 best FG-producers were further characterised by western blot and ELISA. Again, the 8 best FG-subclones were further amplified and their FG expression was evaluated in presence and absence of  
25 sodiumbutyrate (2mM) or DMSO (1 or 2%). Six subclones were amplified and cell vials were made and stored at -80°C and liquid N2. Finally, the 3 best FG-subclones were selected. These are CHOK1 FG ° 7.18, ° 13.1 , and ° 37.2.

Westernblot analyses (non-reducing conditions) with monoclonal mAB19 indicated a major band of FG at about 135 kDa. The purified FG protein from  
30 recombinant Baculovirus FG infected cells (UPJOHN) appeared as major broad bands at +/-100kDa and other bands at +/-70kDa under similar blot conditions.

The addition of Sodium butyrate in CHO-FG cell culture medium increased the expression level of FG 3 to 12 fold depending on the subclone and cell culture growth conditions. In particular, subclone CHO-FG 13.1 expressed 8-10 fold more FG protein in the presence of butyrate (WB/ELISA).

5        Expression level determination was performed by ELISA (mAB19 or MoAb AK13) using purified FG baculo protein as standard, as well as by western blot analysis using serial dilution.

      Depending of the ELISA assay and cell culture conditions, the expression level of CHO-FG 13.1 is 5-12 ug of FG/ml after treatment with butyrate. Under  
10       accumulation conditions and medium replacements (3 to 5 days ) yields of 16 to 28 ug of secreted FG protein /ml were obtained.

      CHOK1 FG 13.1 cell line was adapted to grow in suspension and serumfree (S/SF) conditions using a proprietary growth medium. Cell line CHO-FG 13.1 S/SF grown in a medium without butyrate expressed similar yields as the parental adherent  
15       cell line grown in medium with butyrate. The addition of butyrate to CHO-FG 13.1 S/SF media has little effect on production of FG (1.5 to 2 fold increase).

      Long term expression evaluation and preliminary genetic characterisation showed that CHO-FG 13.1 and the S/SF adapted 13.1 cell line were stable, contained intact FG expression cassettes giving rise to one single mRNA band of about 3000  
20       nucleotides long (Southern and Northern analyses). The CHO-FG clone 13.1 S/SF was further used for production of FG antigen.

      The use of alum/MPL/QS21/SUV for the enhancement of the immune response in African Green Monkeys towards the FG protein from RSV (Respiratory Syncytial Virus).

25       The FG protein (fusion protein containing the F- and G- proteins from RSV) was expressed in CHO cells and purified. 20 µg of the purified protein was adsorbed on alum (500 µg) to which monophosphoryl lipid A (MPL: 50 µg) was added. After incubating 30 minutes at room temperature, phosphate buffered saline was added. Then either SUV alone or a mixture of SUV and QS21 (50 µg QS21, SUV containing  
30       250 µg cholesterol and 1 mg DOPC) were added. African green monkeys were

injected three times with these formulations, or with FG on alum alone or FG mixed with MPL, SUV and QS21 in the absence of alum.

Figure 5 below show the RSV neutralising titres and the FG-ELISA titres obtained for each formulation. It is clear that the group alum/MPL/QS21/SUV induces the highest titres.

### **Example 5 Comparison of QS21 / SUV containing formulations with Alum formulation of Hepatitis B vaccine containing SL\* antigen**

#### **10 Introduction**

SL\* was produced in accordance with the procedure set out in European Patent application No. 414374.

An immunogenicity study was conducted in Balb/c mice to compare the humoral responses induced by QS21-SUV containing formulations in presence or not of Al(OH)<sub>3</sub>. MPL dose was 5µg, QS21 5µg, SUV contained 25µg cholesterol and 100µg DOPC.

The experimental protocol is described in Material and Methods. Briefly, mice were immunised intramuscularly in the leg twice at 4 weeks interval with SL\* vaccines containing vehicle, immunostimulants or combinations of both. Anti-HBs humoral responses (IgG and isotypes) were analysed.

The following groups were included in the study:

1. SL\* (2ug) Al(OH)<sub>3</sub> (50 ug)
- 25 2. SL\* (2ug) Al(OH)<sub>3</sub> (50 ug) / MPL / QS21-SUV
3. SL\* (2ug) Al(OH)<sub>3</sub> (50 ug) / QS21-SUV
4. SL\* (2ug) MPL / QS21-SUV

#### **Results**

Humoral responses were measured by Elisa as described in Material and Methods. Two time points were analyses: 28 days after the first injection (28 post I) and 14 days following the booster injection (14 post II).

Post I and post II anti-HBs response analysed on pooled sera are presented in Figure 6.

These data show that in primary response, comparable antibody titers are induced by all formulations containing QS21-SUV while a weaker response is observed when Al(OH)<sub>3</sub> alone is used.

In secondary response, the lowest antibody response was also induced by Al(OH)<sub>3</sub> containing vaccine. However, all formulations containing QS21-SUV did not behave the same way.

The two formulations containing Al(OH)<sub>3</sub> QS21-SUV (+/- MPL) induced the strongest antibody response (2x higher than MPL / QS21-SUV).

Although no statistical analysis has been performed, results on individual sera confirm this observation.

The combination of Al(OH)<sub>3</sub> and QS21-SUV (+/- MPL) also qualitatively affects the immune response as shown by the isotypic profile of the humoral response (Figure 7).

Al(OH)<sub>3</sub> induces a clear TH2 type of immune response (only 3 % IgG2a) whereas Al(OH)<sub>3</sub> / QS21-SUV (+/- MPL) formulations induce up to 46% IgG2a.

### Conclusion

The combination of Alum with QS21-SUV (+/-MPL) induces higher antibody titers than formulations containing vehicle or immunostimulants alone.

### Material and Methods

#### Immunisations

10 groups of 5 female Balb/c mice (6-8 weeks) were immunised intramuscularly in the leg (gastrocnemien) twice at 4 weeks interval with 50µl vaccine containing 2 µg SL\* formulated in Al(OH)<sub>3</sub>(50 ug equivalent Al<sub>3</sub>+), Al(OH)<sub>3</sub> / QS21-SUV, Al(OH)<sub>3</sub> / MPL / QS21-SUV, MPL / QS21-SUV. A dose of 5 ug of immunostimulants was used.

Animals were bled on day 28 (28 post I) and 42 (14 post II) for antibody determination by Elisa.

### Formulations

5            Components batches used.

### Formulation process

SL\* (2ug) is adsorbed or not for 15 min on 50 ug of water diluted  $\text{Al}(\text{OH})_3$ .

10           If needed, 5 ug of MPL is added to the preparation as a suspension of 100 nm particles (MPL out) for 15 min. If needed, ten fold concentrated buffer is added before adding 5 ug of QS21 mixed with liposomes in a weight ratio QS21 / Cholesterol of 1/5.

Thiomersal is added to the formulations 15 min after QS21/SUV addition.

15           Formulations containing QS21-SUV are buffered with PBS pH 7.4 and the others are prepared in PBS pH 6.8

### Serology

Quantitation of anti-HBs antibody was performed by Elisa using HBs (Hep286) as coating antigen. Antigen and antibody solutions were used at 50 ul per well. Antigen was diluted at a final concentration of 1 ug/ml in PBS and was adsorbed overnight at 4°C to the wells of 96 wells microtiter plates (Maxisorb Immuno-plate, Nunc, Denmark). The plates were then incubated for 1 hr at 37°C with PBS containing 1% bovine serum albumin and 0.1% Tween 20 (saturation buffer). Two-fold dilutions of sera (starting at 1/100 dilution) in the saturation buffer were added to the HBs-coated plates and incubated for 1 hr 30 min at 37°C. The plates were washed four times with PBS 0.1% Tween 20 and biotin-conjugated anti-mouse IgG1, IgG2a, IgG2b or a mix of the three antibodies (Amersham, UK) diluted 1/1000 in saturation buffer was added to each well and incubated for 1 hr 30 min at 37°C. After a washing step, streptavidin-biotinylated peroxidase complex (Amersham, UK) diluted 1/5000 in saturation buffer was added for an additional 30 min at 37°C. Plates were washed as above and incubated for 20 min with a solution of o-phenylenediamine (Sigma) 0.04%  $\text{H}_2\text{O}_2$  0.03% in 0.1% tween 20 0.05M citrate buffer pH4.5. The reaction was



stopped with H<sub>2</sub>SO<sub>4</sub> 2N and read at 492/620 nm. ELISA titers were calculated from a reference by SoftmaxPro (using a four parameters equation ) and expressed in EU/ml.

**Claims**

1. An adjuvant composition comprising alum, an immunologically active saponin fraction, and a sterol.
- 5 2. An adjuvant composition according to claim 1 wherein the immunologically active saponin fraction is QS21.
- 10 3. An adjuvant composition according to claim 1 or 2 wherein the saponin is associated with liposomes comprising a phospholipid and a sterol.
4. An adjuvant composition according to any one of claims 1 to 3 wherein the sterol is cholesterol.
- 15 5. An adjuvant composition according to claim 2, 3 or 4 wherein the ratio of QS21:sterol is from 1:100 to 1:1.
6. An adjuvant composition according to any of claims 1 to 5 which further contains 3-de-O-acylated monophosphorly lipid A.
- 20 7. A vaccine comprising an adjuvant of claim 1 to 6 and an antigen.
8. A vaccine composition as claimed herein comprising an antigen or antigenic composition derived from any of Human Immunodeficiency Virus, Feline  
25 Immunodeficiency Virus, Varicella Zoster virus, Herpes Simplex Virus type 1, Herpes Simplex virus type 2, Human cytomegalovirus, Hepatitis A, B, C or E, Respiratory Syncytial virus, human papilloma virus, Influenza virus, Hib, Meningitis virus, Salmonella, Neisseria, Borrelia, Chlamydia, Bordetella, Plasmodium or Toxoplasma, adjuvanted with a composition of claim 1 to 6.
- 30 9. A vaccine as claimed in any of claim wherein the antigen is a tumour antigen.
10. A vaccine as claimed in claim 8, wherein the antigen is selected from the group SL\* derived from Hepatitis B, HSV gD<sub>2</sub>t, or an RSV FG chimeric protein.
- 35 11. Use of composition as defined in any of claims 1 to 6 for the manufacture of a vaccine for the prophylactic treatment of viral, bacterial, or parasitic infections.

12. Use of composition as defined in any of claims 1 to 6 for the manufacture of a vaccine for the immunotherapeutic treatment of viral, bacterial, parasitic infections or cancer.
- 5 13. A method of treating a mammal suffering from or susceptible to a pathogenic infection comprising the administration of a safe and effective amount of a composition according to any of claims 1 to 5.
- 10 14. A method of treating a mammal suffering from cancer comprising the administration of a safe and effective amount of a composition according to any of claims 1 to 5.
- 15 15. A process for making a vaccine composition according to claim 1 comprising admixing alum, an immunologically active saponin fraction and cholesterol with an antigen or antigenic composition.

Figure 1 of 7

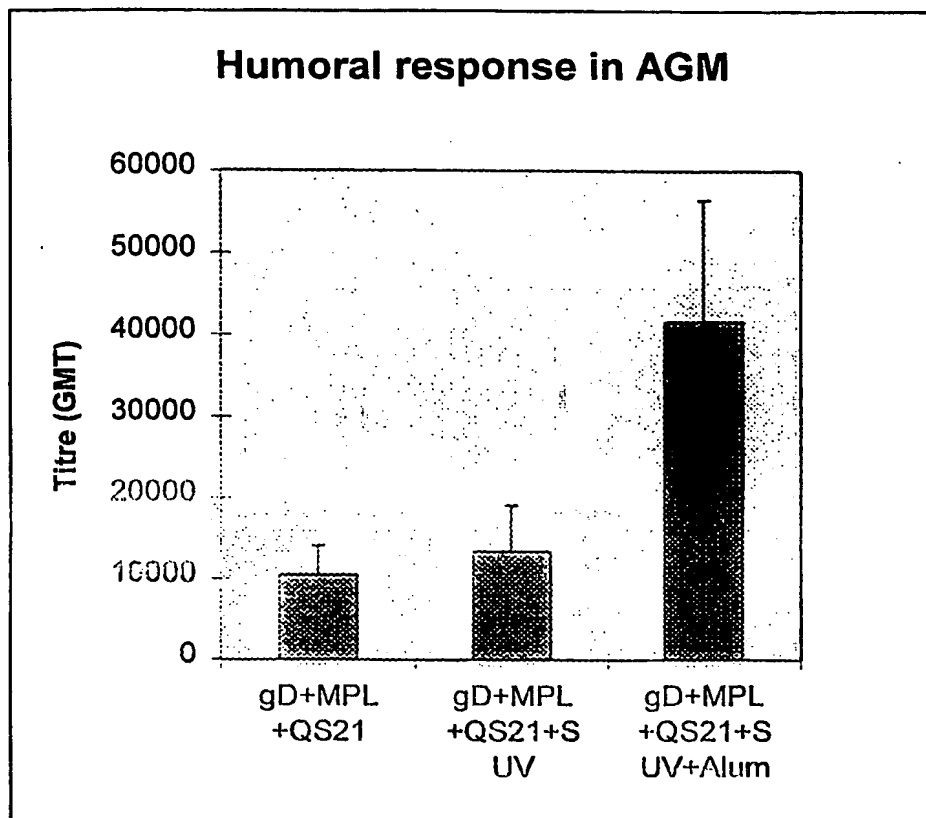
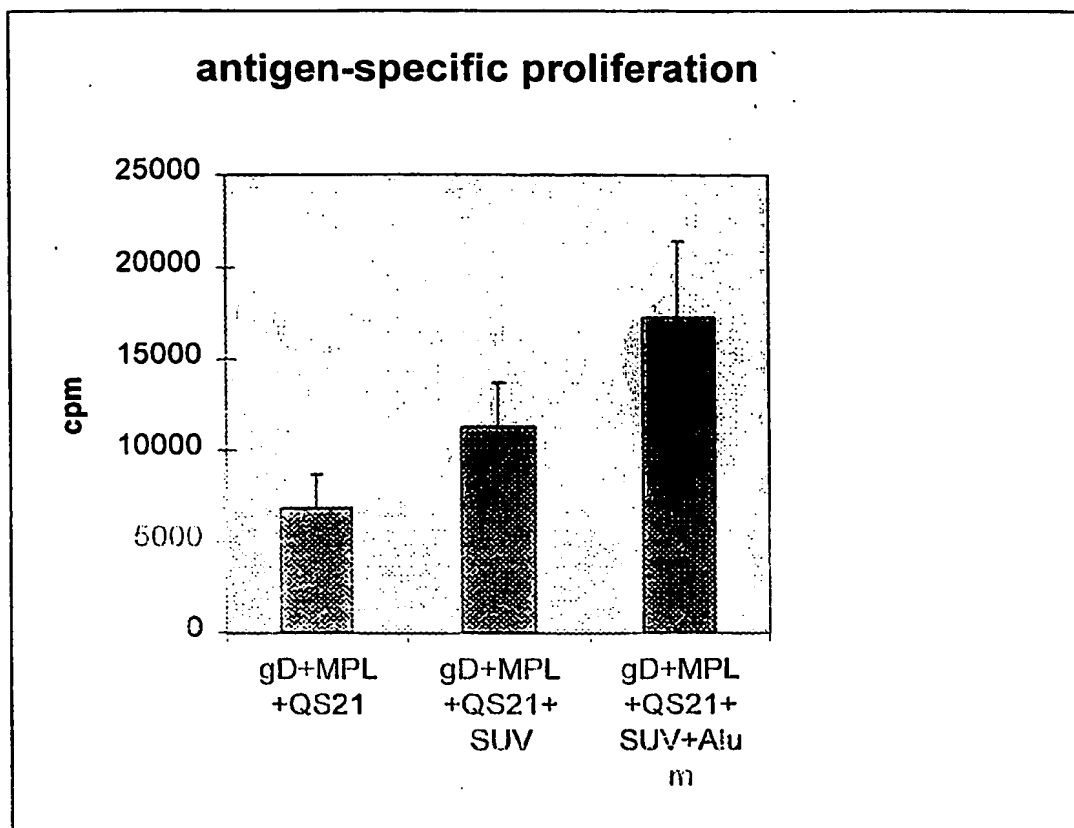
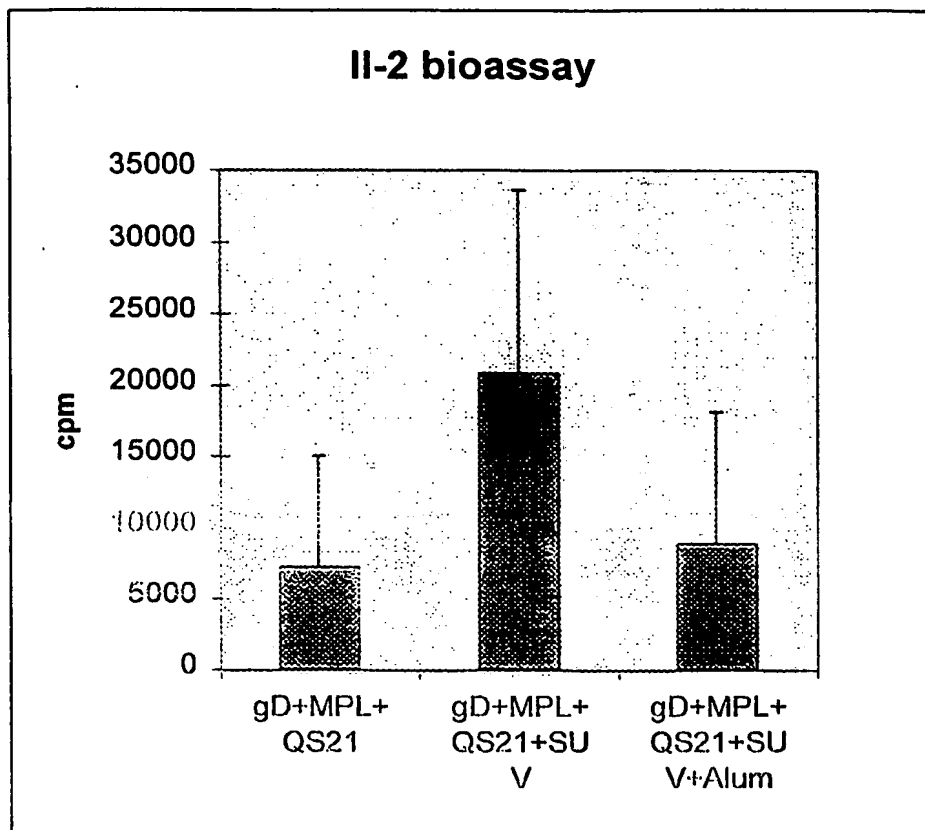


Figure 2 of 7



Antigen specific proliferation was measured by stimulation in vitro with gD coupled to microbeads, and expressed as CPM of 3H-TdR incorporated.

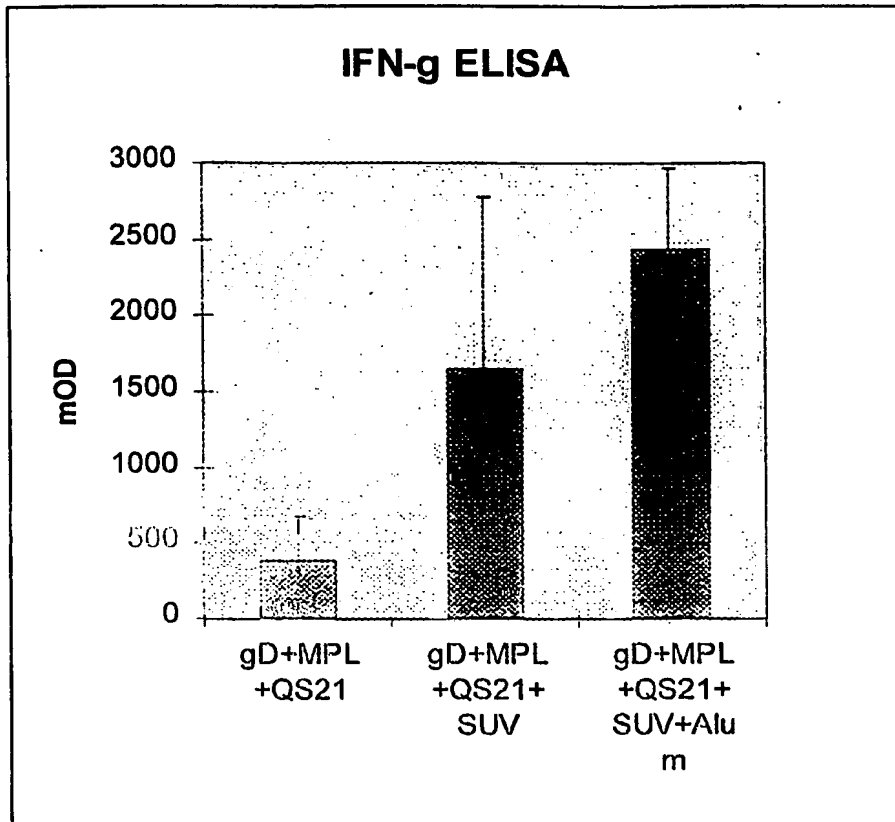
Figure 3 of 7



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The interleukin-2 was measured with a bioassay (incorporation of  $^3\text{H}$ -TdR by an indicator cell line).

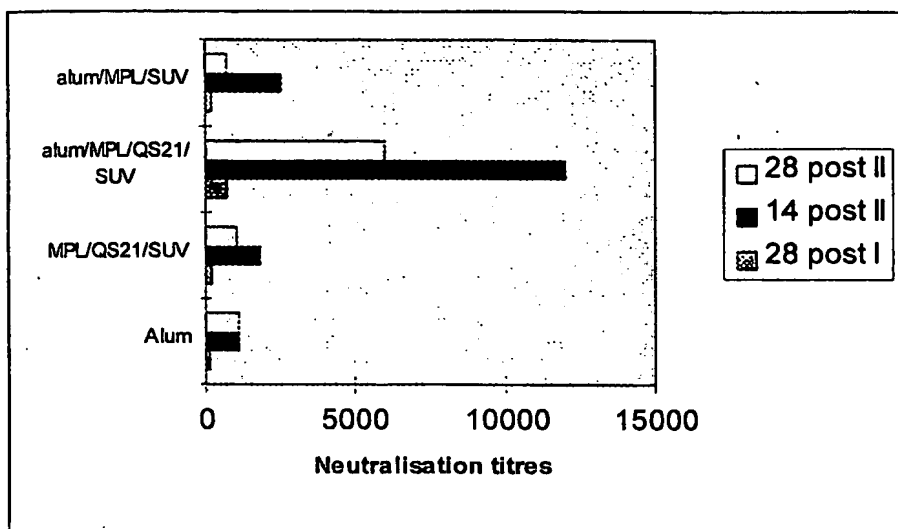
Figure 4 of 7



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Interferon-gamma was measured using an ELISA.

Figure 5 of 7



5

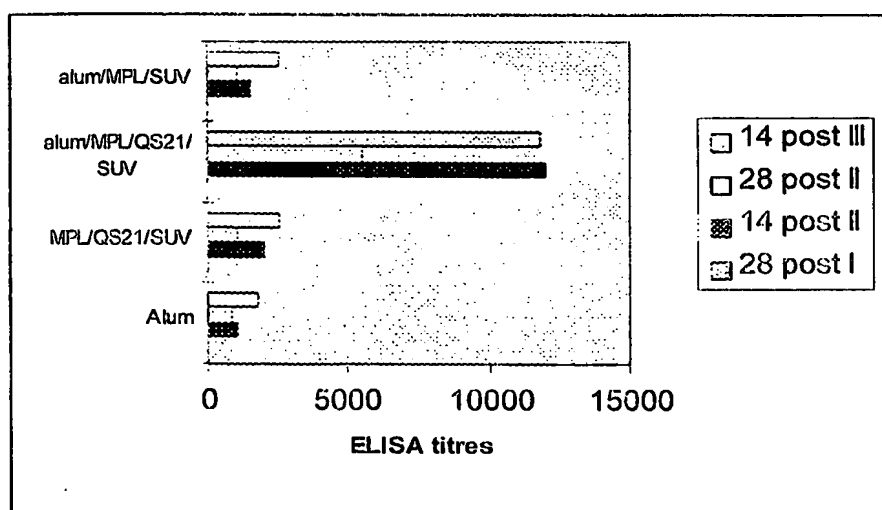




Figure 6 of 7

**Comparison of QS21-SUV containing formulations with Alum formulation**  
**Kinetics of the anti-HBs response (post I/II)**

5

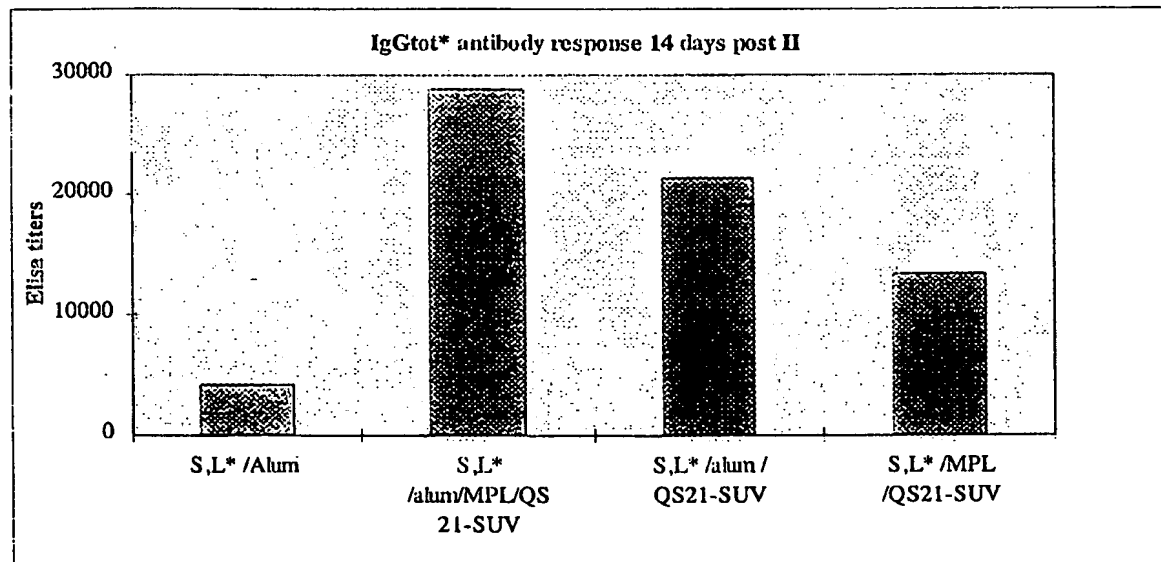
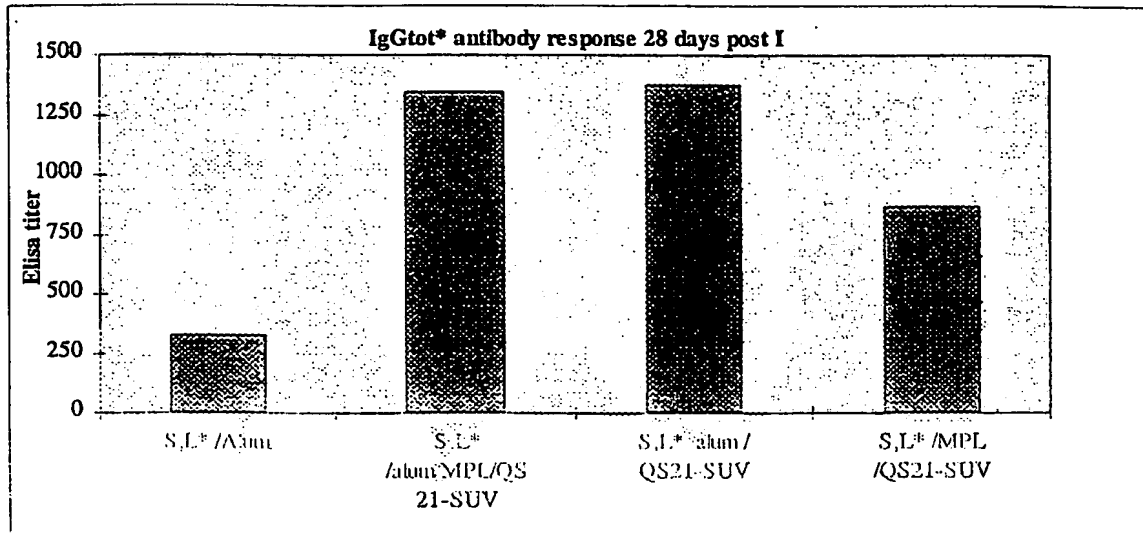


Figure 6 of 7

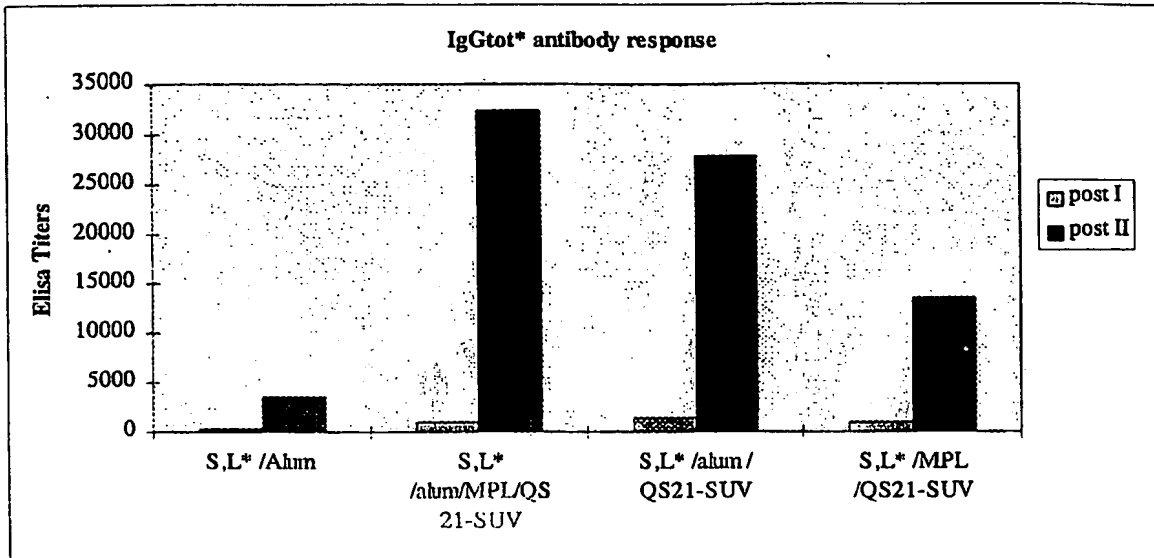
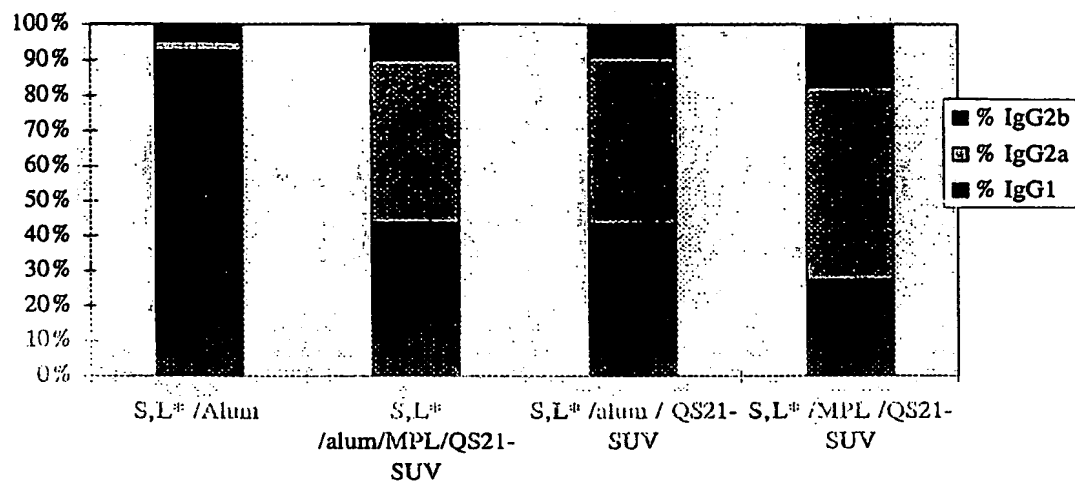


Figure 7 of 7

Comparison of QS21-SUV containing formulations with Alum formulation  
Isotypic profile (post II) anti-HBs response

5

## ISOTIPIC REPARTITION IN POST II



	% IgG1	% IgG2a	% IgG2b
S,L* /Alum	93	3	3
S,L* /alum/MPL/QS21-SUV	44	46	10
S,L* /alum / QS21-SUV	44	47	9
S,L* /MPL /QS21-SUV	27	55	18

# INTERNATIONAL SEARCH REPORT

Int'l. Application No  
PCT/EP 97/05578

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 6 A61K39/39 A61K39/02 A61K39/12

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 93 01831 A (IDEC PHARMA CORP) 4 February 1993 see esp. claims ---	1-15
Y	WO 94 22476 A (SMITHKLINE BEECHAM CORP ;ROBERTS DAVID S (US)) 13 October 1994 see the whole document ---	1-15
Y	KENSIL C. R. ET AL.,: "Separation and characterization of saponis with adjuvant activity from Quillaja saponaria molina cortex" JOURNAL OF IMMUNOLOGY, vol. 146, no. 2, - 2 January 1991 pages 431-437, XP002053915 see the whole document --- -/--	1-8, 10-15

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

\* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
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- "P" document published prior to the international filing date but later than the priority date claimed

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- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
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Date of the actual completion of the international search

29 January 1998

Date of mailing of the international search report

17. 02. 98

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# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/EP 97/05578

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 92 06710 A (NEDERLANDEN STAAT) 30 April 1992 see abstract and materials & methods esp. page 11 ---	1-8, 10-15
A	VOGEL F. R.: "Immunologic adjuvants for modern vaccine formulations" ANN. N.Y. ACADEMY OF SCIENCE, vol. 754, - 1995 pages 153-160, XP002053922 see the whole document ---	1-15
P,X	WO 96 33739 A (SMITHKLINE BEECHAM BIOLOG ;GARCON NATHALIE MARIE JOSEPHE (BE); FRI) 31 October 1996 cited in the application see the whole document -----	1-15

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/EP 97/05578

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 13 and 14  
because they relate to subject matter not required to be searched by this Authority, namely:  
Although claims 13 and 14 are directed to a method of treatment (or diagnostic method practised on) the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 97/05578

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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